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Characterization of High-Risk HIV-1 Seronegative Hemophiliacs

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Mechanisms that protect most high-risk HIV-1 seronegative (HRSN) persons are not well understood. Among hemophiliacs from the Multicenter Hemophilia Cohort Study who remained HIV-1 seronegative despite a high (94%) risk for acquisition of HIV-1 infection, only 7/43 were homozygous for the protective CCR5 Δ32 polymorphism. Among the remainder, neither CCR5 density nor β-chemokine production, nor in vitro susceptibility to infection with the HIV-1 isolate JR-FL could distinguish HRSN hemophiliacs from healthy controls. When compared to lymphocytes of healthy controls not at risk for HIV-1 infection, diminished spontaneous lymphocyte proliferation was seen in lymphocytes of HRSN hemophiliacs as well as in lymphocytes of hemophiliacs not at risk for HIV-1 infection. Surprisingly sera/plasmas obtained from high-risk HIV-1 seropositve hemophiliacs prior to seroconversion more often contained alloreactive antibodies than date-matched sera/plasmas obtained from HRSN hemophiliacs. Thus alloreactivity may predispose to acquisition of HIV-1 infection after parenteral exposure.

Key Words: **Multicenter Hemophilia Cohort Study; CCR5; β-chemokines; HLA; HIV-1; alloreactive antibodies.**

INTRODUCTION

Not all persons exposed to the human immunodeficiency virus type-1 (HIV-1) through sexual contact or parenterally through contaminated blood products become infected. Fewer than 15% of wives and regular sexual partners of HIV-1-infected men with hemophilia in the Multicenter Hemophilia Cohort Study (MHCS) developed HIV-1 infection despite frequent unprotected sexual intercourse (1). Similarly, Peterman *et al.* reported 24 HIV-1 seronegative women who had each reported more than 100 unprotected intercourse episodes with their HIV-1-infected husbands (2). Luscher *et al.* found that 5% of heavily exposed Nairobi prostitutes escaped HIV-1 infection despite repeated exposure to HIV-1-infected partners (3). Similarly, 6% of heavily treated hemophiliacs in the Multicenter Hemophilia Cohort Study escaped HIV-1 infection despite apparent exposure to contaminated antihemophilic factor concentrates (4).

To date, only a genetic polymorphism in the CC-chemokine receptor CCR5, which serves as an HIV-1 coreceptor, has been shown to provide clear protection from acquisition of HIV-1 infection. A 32-bp deletion in this gene (CCR5 Δ32) results in a truncated nonfunctional protein. The prevalence of homozygosity for the CCR5 Δ32 polymorphism is increased in Caucasians at high risk for HIV-1 infection who are uninfected (5–12), and peripheral blood cells obtained from these persons are resistant to infection with R5-tropic HIV-1 isolates (7, 8). HIV-1 infection with X4-tropic HIV-1 isolates has been reported very rarely in persons homozygous for CCR5 Δ32 (12, 13). Thus, subjects homozygous for the CCR5 Δ32 genotype are highly but not absolutely resistant to HIV-1 infection. Another CCR5 open reading frame mutation that also results in a truncated protein also may confer resistance to HIV-1 infection (14). Other putative protective mechanisms, such as immune recognition of HIV-1 peptides (15–18), the presence of rare HLA alleles (3), and heightened production of HIV-1-inhibiting β-chemokines (19) have been suggested as possibly protective, but these mechanisms are less firmly established.

Among hemophiliacs within the MHCS who were heavily treated with non-heat-treated clotting factor concentrates between 1978 and 1985, Kroner *et al.* (1994) found that the risk of acquiring HIV-1 infection

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was dependent upon both the nature of the treatment (Factor VIII concentrate was associated with higher risk of HIV-1 infection than Factor IX concentrate) and intensity of treatment (4). Only 6% of hemophiliacs heavily treated with Factor VIII concentrate (>20,000 Units/year or >100,000 Units between 1978 and 1985) or Factor IX concentrate (>50,000 Units/year or $>10^6$ Units during this time) remained HIV-1 seronegative (4).

In the present study, we performed careful analyses of blood samples obtained from high-risk HIV-1 seronegative (HRSN) hemophiliacs in the MHCS to characterize this cohort more completely and to explore potential mechanisms for *in vivo* resistance to HIV-1 infection. Our results demonstrate that the protective CCR5 Δ32 polymorphism was seen only in a minority (16%) of HRSN hemophiliacs. Among the remaining HRSN, neither *in vitro* susceptibility to infection with HIV-1 isolates (JR-FL and NL4-3), nor β-chemokine production, CCR5 density, or lymphocyte activation status could distinguish HRSN hemophiliacs from controls. Surprisingly, HRSN hemophiliacs were less likely to have alloreactive antibodies than were high-risk hemophiliacs who later acquired HIV-1 infection.

MATERIALS AND METHODS

Patients and controls. Hemophiliacs at risk for HIV-1 infection were identified from the MHCS. Forty-three patients were identified as HRSN hemophiliacs; each was a heavy user of Factor VIII (>20,000 U/year or >100,000 U of non-heat-treated clotting factor between 1978 and 1985). The predicted risk for HIV-1 infection among these hemophiliacs was 94% (4). Serum samples were repeatedly nonreactive with HIV-1 antigens by EIA, RIA, and immunoblot. Controls for the functional studies were healthy laboratory and health care workers not at risk for HIV-1 infection. For the case-control study of alloreactivity, controls were hemophiliacs within the MHCS who ultimately seroconverted and were matched to the HRSN hemophiliacs for age, intensity of therapy prior to seroconversion, and availability of date-matched serum or plasma samples. For the lymphocyte proliferation studies, 6 HIV-1 seronegative hemophiliacs who had been heavily treated with only heat-treated Factor VIII concentrates and thus were not at risk for HIV-1 infection also served as controls.

Cell preparation and culture conditions. Whole blood drawn into sodium heparin-containing tubes (Becton–Dickenson, Franklin Lakes, NJ) from both HRSN hemophiliacs and healthy controls was either prepared on site or shipped overnight before preparation. Peripheral blood mononuclear cells (PBMC) were purified by Ficoll–Hypaque (Pharmacia, Piscataway, NJ) density sedimentation and were cryopreserved in 90% fetal bovine serum (FBS, Summit Biotechnologies, Fort Collins, CO) and 10% dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO). Frozen PBMC were thawed, washed gently, and cultured overnight in a polypropylene culture tube in complete medium (CM) consisting of RPMI 1640 (BioWhittaker, Inc., Walkersville, MD) supplemented with 20% heat-inactivated FBS, 10 mM Hepes (BioWhittaker), 10 mM L-glutamine (BioWhittaker), 100 U/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker) at 37°C in a humidified 5% CO_2-enriched incubator before functional studies were performed.

DNA preparation and CCR5 genotyping. One hundred thousand PBMC were pelleted and stored at −70°C. DNA was purified using the QiaAmp Blood kit (Qiagen, Santa Clairita, CA). PCR amplification was performed in a solution (25 μl) containing 2.5 pmol of the appropriate positive-strand and negative-strand primers; 67 mM Tris–HCl (pH 8.8); 6.7 mM $MgSO_4$; 16.6 mM $(NH_4)2SO_4$; 10 mM 2-mercaptoethanol; 100 μM dATP, dGTP, dCTP, and dTTP; 2.5 units of thermostable DNA polymerase (PE Applied Biosystems, Foster City, CA), and 10–50 ng of purified human genomic DNA. Oligonucleotide primers used to direct amplification of CCR5 ORF products (weight, 312 bp; Δ32, 280 bp) include 62000^+ 5′-GTC TTC ATT ACA CCT GCA GCT CTC-3′ and 62311^- 5′-GGT CCA ACC TGT TAG AGC TAC TGC-3′. Amplicons for the CCR5 ORF were visualized on 2% agarose gels following electrophoresis in 1× TBE and stained with a 1:10,000 dilution of SYBR Gold (Molecular Probes, Eugene, OR) and detected using a Storm 860 scanner (Molecular Dynamics, Sunnyvale, CA).

Lymphocyte proliferation. PBMC were cultured in quadruplicate round-bottom microtiter wells at a concentration of 10^5 cells/0.1 ml CM. Lymphocyte proliferation was assayed in medium alone or medium supplemented with increasing concentrations of PHA (Sigma) and IL-2 (Cellular Products, Inc., Buffalo, NY) (0.15/0.15, 0.31/0.31, 0.63/0.63, 1.25/1.25, 2.5/2.5, 5/5, and 10/10 μg/ml PHA/%IL-2, respectively). After 3 days of culture, 1 μCi of [^{3}H]thymidine (ICN Pharmaceuticals, Inc., Cosa Mesa, CA) was added to all wells. Cells were harvested 18 h later using a semiautomated cell harvester and [^{3}H]thymidine incorporation was assayed using automated gas scintillation spectrometry. Lymphocyte proliferation was recorded in counts per minute, and 10 and 50% of maximal stimulation was defined for each individual based on the analysis of the stimulation response curves. It should be noted that for all subjects, although peak proliferation varied, the concentrations of stimuli that resulted in 10 and 50% of maximal stimulation were remarkably consistent (0.4 μg/ml PHA, 0.4% IL-2 and 1.25 μg/ml PHA, 1.25% IL-2, respectively).

Viral stocks and in vitro infections. HIV-1 laboratory isolates NL4-3 and JR-FL were obtained from the AIDS Reference and Reagent Program (Rockville, MD) and were propagated in PHA blasts obtained from healthy controls using standard methodologies (20). Culture supernatant was analyzed for HIV-1 p24 antigen concentration by ELISA (Coulter, Miami, FL) and the $TCID_{50}$ of each virus isolate was determined by limiting dilution using PHA blasts.

Infectivity assays were performed as follows: PBMC were stimulated at 10 and 50% of maximal stimulation conditions (0.4 μg /ml PHA, 0.4% IL-2 and 1.25 μg /ml PHA, 1.25% IL-2, respectively). After 48 h, cells were centrifuged and incubated for 2 h with the HIV-1 isolates NL4-3 or JR-FL at a m.o.i. of 0.1. The cells were washed three times and resuspended in CM supplemented with IL-2 (0.4% for 10% or 1.25% for 50% maximal stimulation). One hundred thousand cells in 300 μl CM were plated in quadruplicate microtiter wells. Supernatants were collected and replaced with fresh CM at 1, 3, 5, 7, and 11 days postinfection and were analyzed for HIV-1 p24 antigen concentration by ELISA (Coulter).

β-Chemokine production. PBMC were incubated at a concentration of 10^6 cells/ml at 10, 50, or 100% of optimal stimulation conditions or without stimulation for 48 h. Supernatants were assayed for MIP-1α, MIP-1β, and RANTES by ELISA (R&D Systems, Minneapolis, MN).

CCR5 expression. PBMC were incubated without stimulation in CM for 24 h to allow for recovery from cryopreservation. Cells were washed and then resuspended in CM at 1×10^6 cells/ml and cultured for 48 h in a multiwell culture plate. Cells were removed from the culture wells by pipetting up and down while the wells were gently scraped with the pipet tip. Cells were then were centrifuged at 400*g*, washed, and resuspended in 200 μl of PBS. Recovery was $\geq$70%. PBMC were incubated with monoclonal antibodies CD4-PerCP/CD14-FITC/IgG_1-PE, CD4-PerCP/CD14-FITC/CCR5-PE (Pharmingen, San Diego, CA) for 30 min at room temperature, then washed twice, fixed with 1% paraformaldehyde in PBS, and analyzed immediately using a FACScan (Becton–Dickenson, San Jose, CA). The monoclonal antibody directed against the chemokine receptor CCR5 was labeled with fluorochrome at a ratio of 1 molecule of phycoerythrin per antibody molecule. Known ratios of QuantiBRITE-PE beads (Becton–Dickenson) were analyzed using the same instrument settings as used in the assay. The geometric mean fluorescence for the control IgG2a-PE was subtracted from the geometric mean fluorescence of the CCR5-PE-positive cells and was converted into the number of PE molecules bound per $CD4^+$ T lymphocyte and $CD14^+$ monocyte (21). Monocytes and lymphocytes were analyzed separately using CELLQuest software (Becton–Dickenson) collecting at least 10,000 events for each analysis.

HLA class I and class II reactive antibodies. Serum or plasma samples that had been drawn and frozen 6–9 months before seroconversion from MHCS hemophiliacs who later acquired HIV-1 infection and age-matched, therapy-matched, and time-matched samples obtained from the HRSN hemophiliacs were examined for HLA-reactive antibodies using microcytoxocity assays (Lambda Cell Trays, One Lambda, Inc., Canoga Park, CA). The test wells were scored as positive or negative based upon the frequency of cell death using the American Society of Histocompatibility and Immunogenetics (ASHI) reading standard (22).

RESULTS

Study subjects. Forty-three HRSN hemophiliacs and 28 matched hemophilic seroconverters comprised the study population for alloreactivity studies, and 25 HRSN hemophiliacs, 6 hemophiliacs not at risk for HIV-1 (NRSN), and 19 age-matched healthy controls comprised the study populations for other functional studies (Table 1). Age and sex were comparable within each study population and their controls with the exception of the NRSN hemophiliacs, who had a median age of 15. Ages are listed for the dates of sample acquisition; the alloreactivity studies were performed using samples obtained between 1978 and 1987, while the functional studies were performed on samples obtained between 1995 and 1998. Demographics of the study groups were comparable. The predicted overall risk for seroconversion based upon treatment history was comparable in the HRSN hemophiliacs and in the hemophiliacs who were seroconverters.

CCR5 genotype. Among the HRSN hemophiliacs, 7 of 43 (16%) were homozygous for the CCR5 Δ32 polymorphism and 7 of 43 (16%) were heterozygous (Table 2). Among the seroconverters who were tested, 4 of 19 (21%) were heterozygous for the CCR5 Δ32 polymorphism and none were homozygous. Among the healthy controls, only 1 of 19 (5%) was heterozygous for the CCR5 Δ32 polymorphism.

Cell surface CCR5 expression. $CD4^+$ T cell expression of CCR5 was measured on unstimulated cultured lymphocytes (Fig. 1). As expected, the seven HRSN homozygous for the CCR5 Δ32 polymorphism had undetectable CCR5 expression on $CD4^+$ T cells (not shown) and the two HRSN hemophiliacs heterozygous for CCR5 Δ32 had low levels of CCR5 expression averaging 286 molecules per cell (not shown). Therefore comparative analysis was restricted to PBMC of persons with wild-type CCR5 open reading frame se-

TABLE 1

Demographics of Study Subjects

	HIV-1 status			Healthy controls
	Seroconverters	HRSN	NRSN	
Age mean ± SD[a]				
Alloreactivity studies	25 ± 16 (n = 28)	26 ± 14 (n = 43)		
Cellular assays		43 ± 14 (n = 25)	15 ± 2 (n = 6)	39 ± 13 (n = 19)
Sex				
Male/female	28/0	42/1	6/0	19/0
Race				
White non-Hispanic	23	41	3	19
Black non-Hispanic	2	0	2	
Hispanic	2	1	0	
Other	1	1	1	
Antihemophilic factor usage				
Factor VIII high	25	43	6	NA
Factors VIII and IX	3			
Predicted risk for HIV-1 infection	94%	94%	0	0

[a] At the time of blood sampling, during the years 1978–1987 for alloreactivity studies and 1995–1998 for cellular assays.

quences. $CD4^+$ T cell surface CCR5 densities were comparable among HRSN hemophiliacs and healthy controls (1080 vs 1019 molecules/cell). The percentages of $CD4^+$ T cells that expressed CCR5 were also comparable among HRSN and controls (mean values of 14 ± 9 and 10 ± 5%, respectively). The density of CCR5 molecules on cultured $CD14^+$ monocytes of HRSN and controls was similar averaging 9714 molecules per cell in the HRSN and 11,204 molecules per cell among the healthy controls. Examination of peak shifts on flow histograms demonstrated that essentially all $CD14^+$ cells from both the HRSN and healthy controls expressed CCR5 (data not shown).

Lymphocyte proliferation. The "activation state" of patients' and controls' PBMC was tested by measurement of thymidine incorporation in the absence of stimulation and after exposure to graded concentrations of PHA and IL-2. Unstimulated PBMC obtained from HRSN hemophiliacs had less spontaneous thymidine incorporation than did unstimulated PBMC from healthy controls (152 cpm vs 448 cpm, $P < 0.01$, Student's t test, Fig. 2). In addition, at the three lowest concentrations of PHA and IL-2, PBMC from HRSN had lower levels of thymidine incorporation than did PBMC from healthy controls ($P < 0.02$, Student's t test, for each comparison). Although at all conditions tested, thymidine incorporation by PBMC from HRSN hemophiliacs and NRSN hemophiliacs tended to be lower than that of controls' PBMC, the difference in the magnitude of these responses diminished as the concentrations of PHA and IL-2 approached maximal stimulation levels. The diminished activation state of PBMC

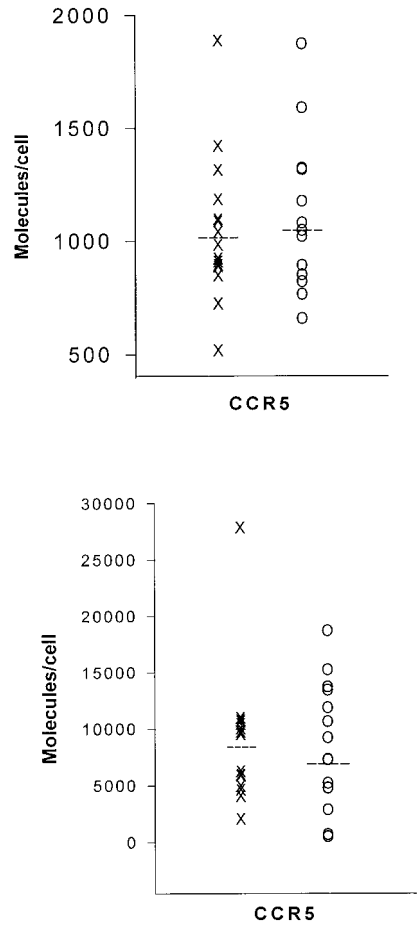

FIG. 1. CCR5 density on $CD4^+$ lymphocytes (top) and $CD14^+$ monocytes (bottom). PBMC were incubated for 48 h without stimulation. Directly conjugated monoclonal antibodies were used to identify both $CD4^+$ T cells and $CD14^+$ monocytes and the β-chemokine receptor CCR5; chemokine receptor density was quantified using fluorescent beads as standards (see Materials and Methods). X represents healthy controls, O represents HRSN who had CCR5 wild-type ORF sequences, and bars represent means for each group. Comparisons were made by using Student's t test.

TABLE 2

CCR5 Genotype

	CCR5 genotype			
	+/+	+/Δ32	Δ32/Δ32	Unknown
HRSN (n = 43)	29	7	7	0
Seroconverters (n = 28)	15	4	0	5
NRSN (n = 6)	2	0	0	4
Healthy controls (n = 24)	23	1	0	0

Note. (+/+) Homozygous wild type, (+/Δ32) heterozygous, and (Δ32/Δ32) homozygous for a 32-bp deletion in the CCR5 ORF.

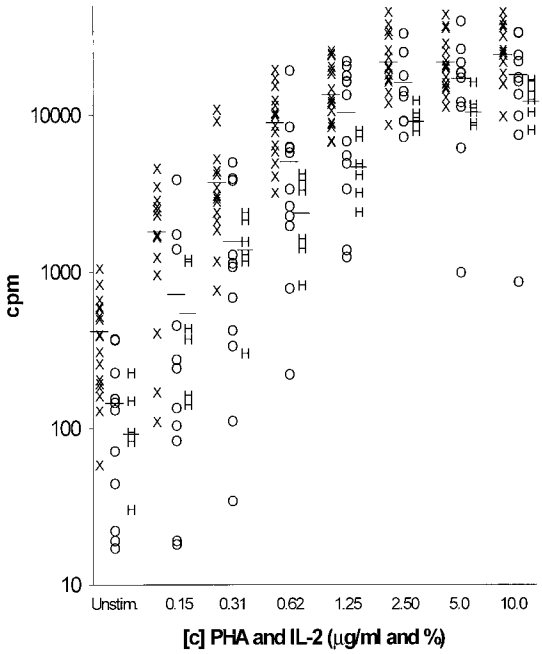

FIG. 2. Lymphocyte proliferation. PBMC were incubated without stimulation or with increasing concentrations of PHA (0–10 μg/ml) and IL-2 (0–10%) for 72 h; [^{3}H]thymidine incorporation is expressed in cpm. X represents healthy controls, O represents HRSN hemophiliacs who had wild-type CCR5 ORF sequences, H represents NRSN hemophiliacs, and bars represent the mean.

obtained from HRSN hemophiliacs was also seen in PBMC obtained from NRSN hemophiliacs, suggesting that lower activation status is characteristic of hemophilia, its treatment, or its complications. Interestingly, thymidine incorporation by PBMC of hemophiliacs who were homozygous for the Δ32 CCR5 polymorphism was comparable to that of controls' PBMC and lower than the baseline thymidine incorporation of HRSN PBMC ($P < 0.01$, Student's t test, data not shown).

Infectivity. To test the susceptibility of PBMC to HIV-1 infection under standardized conditions of suboptimal stimulation that might better approximate levels of activation achieved *in vivo,* assays of lymphocyte proliferation were first performed using PBMC of each subject with serial twofold dilutions of PHA from 10 to 0.15 μg/ml PHA and serial twofold dilutions of IL-2 from 10 to 0.15% (Fig. 2). Conditions that comprised 10 and 50% of maximal stimulation were then derived from these dose–response curves. Our preliminary findings indicated that when using PBMC obtained from healthy controls, infection was unusual using PHA and IL-2 concentrations that induced less than 10% of maximal stimulation (not shown). Therefore, each subject's cells were tested for susceptibility to *in vitro* infection with HIV-1 after stimulation using PHA and IL-2 concentrations that provided 10% (0.4 μg/ml PHA:0.4% IL-2) and 50% (1.25 μg/ml PHA:1.25% IL-2) of maximal stimulation. As anticipated, PBMC of five HRSN hemophiliacs who were homozygous for the CCR5 Δ32 polymorphism were resistant to infection with the R5 HIV-1 isolate JR-FL, but not to the X4 HIV-1 isolate NL4-3 (Fig. 3). In contrast, PBMC obtained from three HRSN hemophiliacs who were heterozygous for CCR5 Δ32 were susceptible to infection with JR-FL but tended to produce less HIV-1 p24 antigen in culture than PBMC of healthy controls who lacked this polymorphism, averaging 16.6 ± 25.6 ng/ml versus 85.6 ± 74.9 ng/ml ($P < 0.01$, Student's t test) at day 11. Moreover, at 10% of optimal stimulation, PBMC from HRSN hemophiliacs who were heterozygous for CCR5 polymorphism had a delay in the appearance of HIV-1 p24 antigen in the supernatant in comparison to PBMC from HRSN and healthy controls who lacked this polymorphism. Among the remaining HRSN hemophiliacs, the PBMC susceptibility to infection with JR-FL and NL4-3 was comparable to the susceptibility of PBMC obtained from healthy controls both in magnitude of HIV-1 p24 antigen production and in the kinetics of its appearance. Interestingly, PBMC obtained from the HRSN hemophiliacs who were homozygous or heterozygous for CCR5 Δ32 had a delayed appearance and lower peak production of HIV-1 p24 antigen in culture supernatant after infection with the X4-tropic HIV-1 isolate NL4-3 under suboptimal stimulation conditions, but these differences were not significant.

β-Chemokine production. Unstimulated PBMC obtained from HRSN hemophiliac subjects without the CCR5 Δ32 polymorphism produced higher levels of MIP-1α (173 vs 46 pg/ml, $P < 0.05$, Student's t test) than did unstimulated PBMC obtained from healthy controls (Fig. 4). Baseline production levels of MIP-1β and RANTES were comparable among HRSN and healthy controls. At 50% maximal stimulation, however, production of MIP-1α and MIP-1β was greater by controls' PBMC than by the PBMC of HRSN hemophiliacs (13,941 vs 2925 pg/ml MIP-1α, $P < 0.001$, Student's t test) and (11,571 vs 3332 pg/ml MIP-1β, $P < 0.001$, Student's t test) (Fig. 4). At all other conditions, production of β–chemokines by PBMC of HRSN hemophiliacs and healthy controls was comparable.

Alloreactivity. Shown in Table 3 are the frequencies of alloreactive antibodies in sera or plasmas obtained from hemophiliacs who later seroconverted to HIV-1 infection and from HRSN hemophiliacs who were matched to the seroconverters by age, calculated risk of HIV-1 seroconversion (based upon treatment intensity), and by dates of sample acquisition. Among those who later seroconverted, 4 of 28 had antibodies to class I HLA antigens only, 3 had antibodies directed

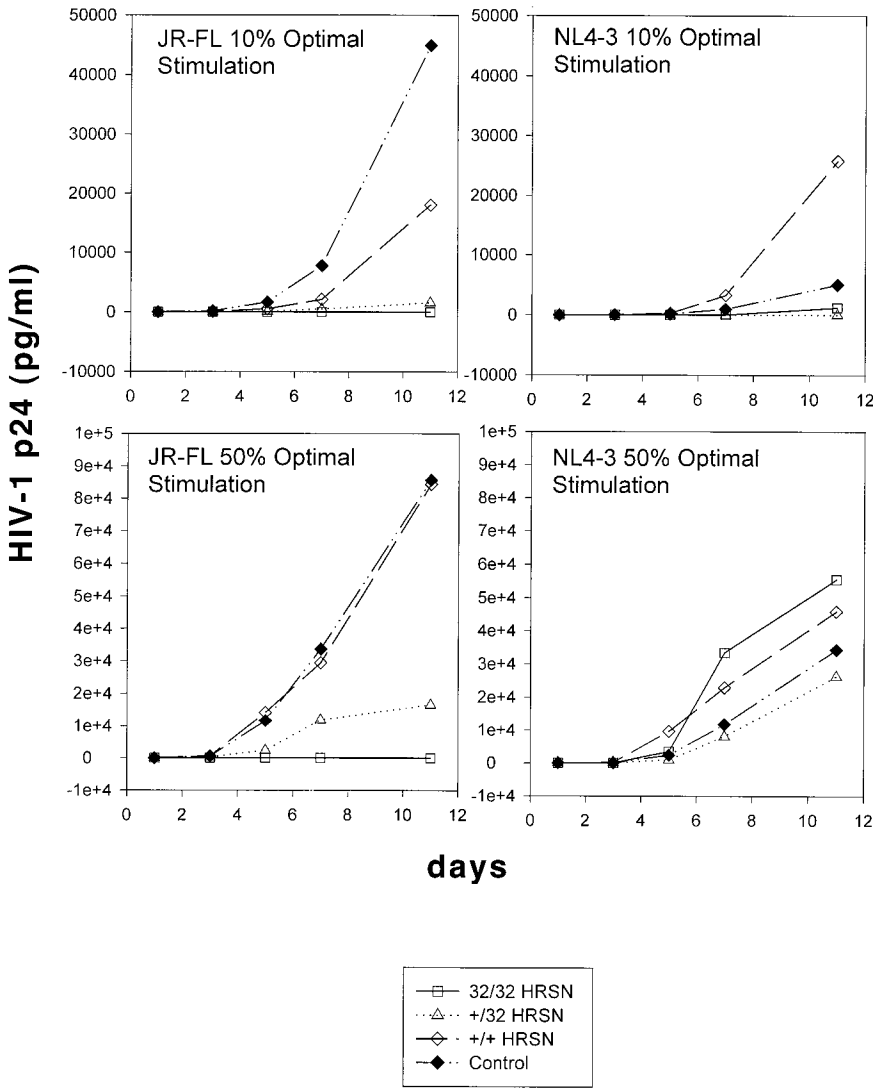

FIG. 3. Infectivity. PBMC were stimulated for 48 h at 10% (0.4 μg/ml PHA and 0.4% IL-2) and 50% (1.25 μg/ml PHA and 1.25% IL-2) of maximal stimulation. Cells were incubated for 2 h with NL4-3 or JR-FL (M.O.I., 0.1) and then washed. Data represent mean HIV-1 p24 antigen concentrations in supernatant. HRSN hemophiliac data shown according to CCR5 open reading frame genotype.

against class II HLA antigens only, and 4 had serum antibodies to both class I and class II antigens. Among the HRSN hemophiliacs, only 1 of 43 had antibodies to class I HLA antigens, 2 had antibodies against class II HLA antigens, and none had antibodies to both. The overall prevalence of alloreactivity was 11/28 (39%) in those who later seroconverted and 3/43 (7%) among the HRSN subjects ($P < 0.01$ by χ^2 test).

DISCUSSION

In this group of HRSN hemophiliacs from the MHCS, 7 of 43 (16%) were homozygous for the CCR5 Δ32/Δ32 polymorphism that provides protection from R5-tropic HIV-1 viruses *in vitro* (23) and *in vivo* (5, 8, 24). The prevalence of this genetic polymorphism is enriched in uninfected but high-risk populations, including those with sexual (25) or parenteral exposure (24). HIV-1 infection has been documented rarely in persons with this trait, but sequence analysis has identified these viruses as compatible with X4-tropic strains (13).

Unstimulated PBMC from HRSN hemophiliacs and NRSN hemophiliacs and PBMC activated with very low concentrations of PHA and IL-2 were less activated to incorporate thymidine than were PBMC of healthy controls. Since low levels of thymidine incorporation also were seen with PBMC of hemophiliacs not at risk for HIV-1 infection, we believe that this phenomenon is a consequence of hemophilia, its treatment, or compli-

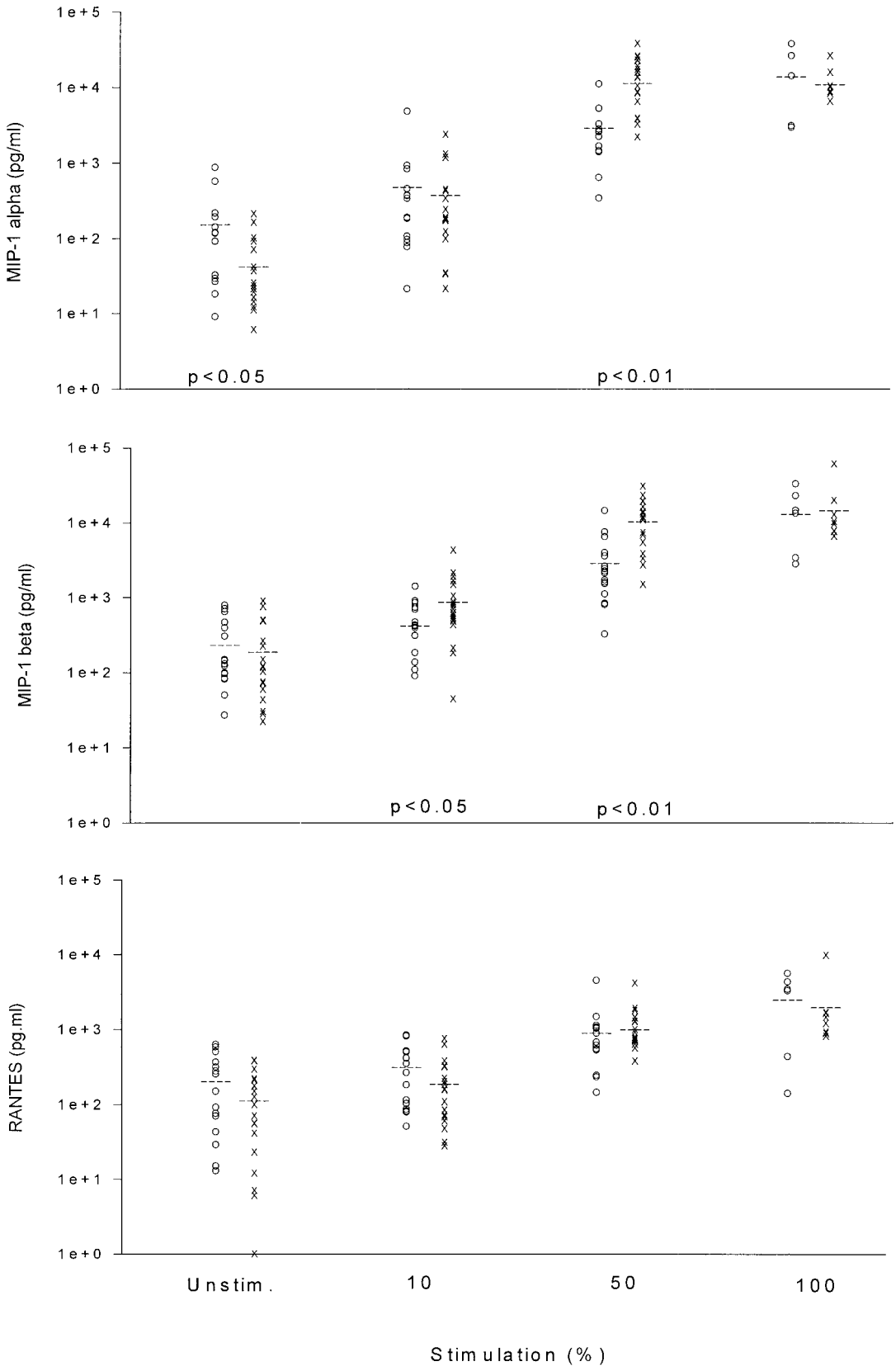

FIG. 4. β-Chemokine production. PBMC were incubated for 48 h without stimulation or at 10% (0.4 μg/ml PHA, 0.4% IL-2) and 50% (1.25 μg/ml PHA, 1.25% IL-2) of maximal stimulation. Chemokine levels in supernatants were measured by ELISA. X represents healthy controls, O represents HRSN who had wild-type CCR5 ORF sequences, and bars represent the means.

TABLE 3

HLA Class I and Class II Alloantibodies

	HRSN	Seroconverters		
Factor concentrate usage (*n*)	VIII (43)	VIII (25)	VIII and IX (3)	Both (28)
Class I only (%)	1 (2)	3 (12)	1 (33)	4 (14)
Class II only (%)	2 (5)	3 (12)	0	3 (11)
Class I and class II (%)	0	4 (16)	0	4 (14)
Total (%)	3 (7)*	10 (40)	1 (33)	11 (39)*

Note. Pre-seroconversion sera/plasmas were obtained from HIV-1 seroconverters and from HRSN hemophiliacs matched to the seroconverters for HIV-1 infection risk and date. Samples were tested for HLA-reactive antibodies by microcytotoxicity assays.

* $P < 0.01$ by χ^2 test.

cations thereof. It is not clear why PBMC obtained from HRSN hemophiliacs with the Δ32 CCR5 polymorphism show activation levels comparable to that of controls' PBMC. Conceivably, complete absence of this chemokine receptor results in compensatory mechanisms for cellular activation that override the effects of hemophilia or its treatment on cellular activation.

When subjects with the CCR5 Δ32 polymorphism were excluded from analysis, cell surface densities of the CCR5 HIV-1 coreceptor on cultured monocytes and $CD4^+$ T cells were comparable in HRSN hemophiliacs and healthy controls.

Zagury *et al*. found that PBMC of exposed uninfected hemophiliacs in the Milan, Italy, cohort produced increased levels of β-chemokines when stimulated with maximal concentrations of PHA and IL-2 (19). As MIP-1α, MIP-1β, and RANTES have potent antiretroviral effects *in vitro* (19, 26) the investigators suggested that resistance in these subjects was immunologically mediated and was reflected in heightened release of β-chemokines in response to mitogenic stimulation (19). Since data generated earlier (27) suggested that PBMC obtained from persons homozygous for the CCR5 Δ32 polymorphism may produce more β-chemokines *in vitro* than PBMC obtained from person with wild-type alleles, we excluded all subjects with the CCR5 Δ32 alleles from the analysis. A restricted analysis was also preferred since persons homozygous for this polymorphism have another defined mechanism for resistance to HIV-1 infection. Using this data set, unstimulated PBMC of HRSN hemophiliacs produced only slightly greater levels of MIP-1α than did PBMC of healthy controls. At all other conditions, β-chemokine production by PBMC of HRSN hemophiliacs was either comparable to or even lower than β-chemokine production by PBMC of healthy controls. It should be noted that β-chemokine production by PBMC of HRSN hemophiliacs who were heterozygous for the CCR5 Δ32 polymorphism was comparable to that of the other HRSN subjects (not shown). Thus, although high-level combinations of all three β-chemokines can limit propagation of HIV-1 *in vitro* (26, 28), our results do not suggest that differences in baseline or stimulated β-chemokine production underlie the apparent protection from HIV-1 infection among HRSN hemophiliacs in the MHCS. Likewise, Fowke *et al*. found that PBMC obtained from HIV-1-resistant women in the Nairobi-based Pumwani Sex Workers Cohort produced significantly less MIP-1α and MIP-1β than did PBMC of low-risk controls (15). We suspect therefore that in these high-risk populations, HIV-1 resistance is not related to increased production of β-chemokines.

Induction of both class I and class II MHC-restricted cell-mediated immune responses in the absence of serologic responses after exposure to HIV-1 has been proposed to be protective (16–18, 29–32). It should be noted that IL-2 responses to HIV-1 peptides had been measured in a subset of the participants in this study and their responses could not be distinguished from those of healthy controls and health care workers (not shown). Since those experiments were performed more than 8 years after last exposure to HIV-1, it is not clear whether these results are reflective of the waning of immunity in the absence of antigenic exposure or whether HIV-1-specific immune responses did not develop in these high-risk subjects.

As anticipated, assays of *in vitro* susceptibility to HIV-1 demonstrated that PBMC obtained from HRSN hemophiliacs homozygous for the CCR5 Δ32 polymorphism were resistant to *in vitro* infection with the R5 isolate JR-FL. PBMC obtained from persons heterozygous for this polymorphism were less supportive of productive HIV-1 infection, as determined by time to detection of HIV-1 antigen in culture and magnitude of antigen production (Fig. 3). Interestingly, PBMC obtained from persons with this polymorphism also were less supportive of productive infection by the X4 isolate NL4-3 under suboptimal stimulation conditions. Review of earlier studies performed among exposed but uninfected persons who were homozygous for CCR5 Δ32 revealed that $CD4^+$ T cell clones prepared from those subjects also tended to support replication of an X4 HIV-1 isolate less well than did $CD4^+$ T cell clones prepared from persons with wild-type CCR5 open reading frame sequences (33). Although Bermejo *et al*. found that activated $CD4^+$ T cells with diminished CXCR4 coreceptor expression had a decreased susceptibility to infection with the NL4-3 HIV-1 isolate (34), this phenomenon does not account for our findings since we did not find diminished CXCR4 density on $CD4^+$ T cells of HRSN hemophiliac patients (not shown). A diminished activation status also is not likely responsible for the delayed appearance of p24 antigen in supernatants of PBMC obtained from persons with the CCR5 Δ32 polymorphism since both

CCR5 Δ32 heterozygotes (whose PBMC had a diminished activation status) and CCR5 Δ32 homozygotes (whose PBMC were as activated as healthy controls' PBMC) showed diminished productive infection with NL4-3. The mechanism underlying this phenomenon is not clear. Excluding these patients, however, there was no difference between the remaining HRSN hemophiliacs and the healthy controls in magnitude of HIV-1 p24 production or time to appearance of HIV-1 antigen in culture supernatants (Fig. 3). In earlier studies, we had shown that PBMC obtained from selected HRSN hemophiliacs at highest risk for HIV-1 infection were less "infectable" than were PBMC obtained from healthy controls (35). Those earlier studies utilized a limiting dilution assay and a pool of clinical viral isolates instead of the characterized strains used in the present study. We do not think that the utilization of clinical isolates better discriminates differences in cellular susceptibilities to HIV-1 infection since the most fit viruses are likely to outgrow in each experiment. We suspect that even though the limiting dilution assay may be more sensitive to subtle heterogeneities in susceptibility to HIV-1 infection than the assay system used in the present study, current *in vitro* assays of cellular infection with HIV-1 that utilize high concentrations of mitogen and interleukin-2 to activate $CD4^+$ T cells provide conditions that are not reflective of susceptibility to HIV-1 infection *in vivo* and for these reasons do not reliably identify the majority of persons who may escape HIV-1 infection after exposure (15). Whether abortive infections in these persons result in the development of both MHC class-I- and class-II-restricted immune responses (16–18, 29, 36) that contribute to protection from subsequent exposures remains to be determined. It should be recognized that all the cellular assays in this study were performed many years after last exposure to HIV-1 and thus susceptibility, activation state, and coreceptor density generated in these studies may not reflect these indices at the time of HIV-1 acquisition risk.

We examined alloreactivity to test the hypothesis that HLA-reactive antibodies might confer some degree of protection from HIV-1 infection. This hypothesis was reasonable since host cell class I and class II HLA antigens are present in high concentration on virion surfaces (37), since antibodies generated against xenogeneic cells used for simian immunodeficiency virus (SIV) propagation were protective against SIV challenge (38, 39), since women who were alloimmunized as a therapy for unexplained recurrent spontaneous abortions developed anti-allo-MHC antibodies that were able to neutralize HIV-1 *in vitro* (31), and since women with rare HLA types were apparently protected from HIV-1 infection even though they had repeated exposure through unprotected sexual intercourse (3). To our surprise HRSN hemophilic sera and plasmas less frequently contained cytotoxic antibodies to HLA class I and class II antigens than did date-matched pre-seroconversion sera obtained from similarly treated hemophiliacs who later acquired HIV-1 infection. Thus, although alloreactive antibodies might permit complement-mediated virus neutralization (40) our findings suggest that HLA-reactive antibodies generated in persons with hemophilia—likely as a result of infusion of plasma products—are not protective against acquisition of HIV-1 infection and, in fact, are associated with a heightened risk of subsequent seroconversion, perhaps through mechanisms such as antibody-mediated enhancement of infection of phagocytic cells (41). In addition, HIV-1 infection also can be enhanced *in vitro* by a complement-mediated, antibody-dependent mechanism and this has been correlated with increased plasma HIV-1 RNA levels and disease progression in HIV-1-infected individuals (42). Complement and antibody binding to virions may increase virion binding to B cells (43) and enhance infection of $CD4^+$ T cells (44). Alternatively, HLA-reactive antibodies may not play an active role in enhancing HIV-1 infection but instead may be reflective of a relatively heightened $CD4^+$ T cell activation in response to alloantigens contained within antihemophilic factor concentrates (41). Allogeneic blood products have been shown to activate HIV-1 expression *in vitro* (45) and modest increases in plasma HIV-1 RNA levels have been reported in HIV-1-infected recipients of whole blood transfusions (46). Thus, alloreactivity may potentially contribute to HIV-1 susceptibility by either directly facilitating infection through immune complex interactions or by creating a state of heightened $CD4^+$ T cell activation.

These studies indicate that there is likely heterogeneity in the mechanisms that protected HRSN hemophiliacs from HIV-1 infection. Only a minority of the HRSN hemophiliacs who did not get infected are homozygous for the protective CCR5 Δ32 polymorphism. Additional studies are needed to determine if diminished alloreactivity or a diminished lymphoid activation state are protective against acquisition of HIV-1 infection. A diminished activation state is characteristic of peripheral blood lymphocytes obtained from persons with hemophilia. This is likely related to the heritable deficiency of Factor VIII or to treatment for hemophilia or its complications. Among persons without the CCR5 Δ32 polymorphism, neither cell surface density of CCR5 nor *in vitro* production of β-chemokines could distinguish HRSN hemophiliacs from healthy controls. Thus we could find no support for the hypothesis that coreceptor inhibition contributes to protection from HIV-1 infection in these persons. In addition, we were unable to demonstrate an intrinsic resistance to HIV infection *in vitro*. However, the *in vitro* assays we utilized to measure susceptibility to

HIV-1 infection employing peripheral blood lymphocytes and high concentrations of mitogen and interleukin-2 may not reflect local conditions or cellular interactions that facilitate productive HIV-1 replication. For this reason, if quantitative differences in susceptibility to HIV-1 infection exist, assays utilizing explanted lymphoid tissue (47, 48) may prove to be more informative. A number of studies have demonstrated the presence of HIV-1-specific immune responses in persons at high risk for HIV infection who have remained uninfected (17, 18). While the presence of these responses may protect against infection after subsequent exposures, this remains to be demonstrated. Additional studies are also needed to identify the factors that permitted the development of immune responses in the absence of productive infection.

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